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Effects of long-term endocrine disrupting compound exposure on *Macaca mulatta* embryonic stem cells

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Abstract

Endocrine disrupting chemicals (EDCs) exert significant effects on health and physiology, many traceable to effects on stem cell programming underlying development. Understanding risk of low-level, chronic EDC exposure will be enhanced by knowledge of effects on stem cells. We exposed rhesus monkey embryonic stem cells to low levels of five EDCs [bisphenol A (BPA), atrazine (ATR), tributyltin (TBT), perfluorooctanoic acid (PFOA), and di-(2-ethylhexyl) phthalate (DEHP)] for 28 days, and evaluated effects on gene expression by RNAseq transcriptome profiling. We observed little effect of BPA, and small numbers of affected genes (119) with other EDCs. There was substantial overlap in effects across two, three, or four treatments. Ingenuity Pathway analysis indicated suppression of cell survival genes and genes downstream of several stress response mediators, activation of cell death genes, and modulations in several genes regulating pluripotency, differentiation, and germ layer development. Potential adverse effects of these changes on development are discussed.

Keywords/phrases

endocrine disruptor; atrazine; perfluorooctanoic acid; phthalate; obesogen; tributyltin; bisphenol A; transcriptome; nuclear programming; gene-environment interaction; developmental origins of disease

Introduction

There are nearly 1000 chemicals listed as potential endocrine disruptor chemicals (EDCs) on The Endocrine Disruption Exchange (endocrindisruption.org). These chemicals are known or suspected of disrupting endocrine signaling with a myriad of downstream effects on development, reproduction, immune function, behavior, cognition, diabetes, cancer, and many other diseases and disorders in individuals and their progeny [1]. The broad spectrum

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of effects, particularly in progeny, reflects the abilities of EDCs to exert significant effects on nuclear programming in the stem cells that contribute to embryogenesis, organogenesis, organ homeostasis, and cancer later in life [2–6].

Understanding the effects of EDCs on stem cells is key for understanding exposure risk and for devising possible strategies for mitigating negative effects of exposures. EDCs are used in a number of industrial processes, including consumer products manufacture and production of herbicides. The widespread industrial use of EDCs is reflected in their presence in serum, urine, amniotic fluid, cord blood, and household dust [1]. Other studies report correlations between these exposures and specific developmental abnormalities and adverse health consequences in humans and other animals [7–20].

One recent study [21] found that species differences in stem cell responses to EDCs can be significant. Consequently, although rodent stem cell and rodent developmental studies provide important information about the potential developmental consequences of EDC exposure, the possibility of species differences needs to be addressed by determining EDC effects in model organisms more closely resembling humans. Some other studies reported that some EDCs affect germ cells and early embryos, but others reported little or no effect [22–32]. Recent studies also illustrate the effects of prolonged low-level exposure on embryonic stem cell (ESC) differentiation [33]. These observations indicate the importance of examining effects on stem cells to better understand potential early developmental consequences of exposure. To explore possible mechanisms underlying EDC effects on early human development, and to establish a foundation for future study in an experimentally tractable animal model closely resembling humans in terms of reproductive physiology, metabolism and developmental mechanisms, we compared effects of long-term culture with each of five EDCs on gene expression patterns in a rhesus monkey embryonic stem cell line. EDCs tested include bisphenol-A (BPA), atrazine (ATR), di-(2-ethylhexyl) phthalate (DEHP), tributyltin (TBT), and perfluorooctanoic acid (PFOA). We applied comparatively low levels of the EDCs, in order to better mimic constant exposures to environmentally relevant concentrations, without complicating influences of acute toxicity, damage to maternal organs, or severe disruptions of maternal endocrine profiles. These compounds were chosen because of their prevalence in the environment and/or in human serum indicating ongoing human exposures, and widespread study. Our results revealed little to no significant effect of BPA with the exposure parameters applied, and significant effects of the other four EDCs. We also observed effects shared between two, three, or four of the latter EDCs, most notably affecting genes related to stress response, cell proliferation, and cell death. Our analysis revealed genes related to specific biological pathways, processes, and functions impacted by these EDCs, and provide novel insight into potential mechanisms whereby early developmental exposure may affect cell reprogramming and thus long-term progeny phenotype.

Methods

Cells and cell culture

A rhesus embryonic stem cell (ESC) line was obtained from the Oregon National Primate Research Center (Beaverton, OR). Oregon Rhesus Macaque Embryonic Stem (ORMES)-6

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line cells were maintained on feeder layers of mouse embryonic fibroblast cells that had been mitotically inactivated. This cell line is one of two available (ORMES-6 and -7, 42XX and 42XY, respectively) for which broad differentiation capacity was reported [34]. ORMES-7 proved more difficult to propagate than ORMES-6, and so was not pursued here. Cells were maintained in Dulbecco's modified Eagle/F-12 medium (DMEM/F12, 11320-033; Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 1% minimum essential medium (MEM) nonessential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 0.1% gentamycin in 60-mm cell culture dishes (Mitalipov et al., 2006). Cultures were incubated at 37°C in 5% CO_2 and 5% O_2. Components for cell culture medium were purchased from Sigma (St. Louis, MO) unless otherwise noted.

For experimental treatments, ESC colonies were cultured in 6-well plates (Falcon 353046), 1 plate for each treatment group. Approximately 40 random colonies per dish were transferred to fresh feeder layers each time ESC colonies were passaged, which occurred every 3 to 4 days. Colonies for passage were taken from a pre-marked, wedge-shaped section of each dish to assure a random sample. Media changes were performed daily (2 mL) and experimental treatments continued for a total of four weeks. A total of six biological replicates were obtained for each treatment group. The cells were harvested and lysed approximately 24 h after the last media change. At harvest, all colonies in each well were cut and lifted, and divided into two microcentrifuge tubes, and centrifuged at 3,000 × g for three min and supernatant removed. Cells were resuspended in 1 mL DPBS with 10% BSA and 25 mM EDTA and centrifuged as above. Supernatant was removed and the remaining cell pellet was lysed in 100 μl PicoPure™ extraction buffer (Thermo Fisher Scientific, Life Technologies, Grand Island, NY).

Chemicals for testing were made as 1000× stocks in methanol and were added to aliquots of ESC maintenance medium up to 48 hours before use and stored at 4°C until use. Chemicals were obtained from Sigma (DEHP: Bis(2-ethylhexyl) phthalate – 47994; PFOA: Perfluorooctanoic acid – 171468; ATR: Atrazine – 45330; TBT: Tributyltin chloride – 442869; BPA: Bisphenol A – 133027). Treatment groups included a vehicle control (methanol) and each of five EDCs at environmentally relevant doses [10 nM BPA (36 nM fetal serum, [35]), 5 μM DEHP (7.7μM pubertal serum, [12]), 30 μM ATR (up to 7 μM drinking water reported, [36]), 100 nM PFOA (average 94 nM 12 year old serum, [37]), and 25 nM TBT (0.17 – 534 adult nM serum, [38]). These concentrations are within the range those reported for human serum, for drinking water, or studies reported for mammalian oocytes and preimplantation stage embryos or pluripotent cells in vitro [22, 23, 27, 39–41]. The concentration of ATR used was ~4-fold higher than the maximum concentration reported in one study in drinking water [36], and one study of occupational human serum level (up to 245 nM [42]). The ATR concentration used affects placenta cell gene expression in vitro [43] and is much lower than the doses (200–300 mg/kg) typically applied in rodent studies to test for reproductive effects. Some of these chemicals exert effects on rhesus monkey gonads, embryo or fetal development, and progeny phenotype, or in human reproductive tissues or stem cells. For example, 10–15 nM BPA affects fetal lung development [44], and affects mammary development [45]. DEHP at 25 μM affects monkey Sertoli cell development [46]. TBT (100 nM) negatively affects human embryonal

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carcinoma cell gene expression and mitochondria [47, 48]. ATR at 200 mg/kg dosing affects gonadogenesis across vertebrate classes [49].

Preparation and sequencing of libraries for RNAseq

RNA was isolated following the PicoPure™ RNA Extraction kit manufacturer protocol, with DNAse digestion to remove any contaminating DNA. To produce libraries for sequencing, 100 ng of each RNA sample (five of the six biological replicates per condition) were processed first using the Ovation RNA-Seq System v2 using Ribo-SPIA™ Technology (NuGen, San Carlos, CA). This was followed by fragmentation to an average of 300 bp using a Covaris-2 sonicator, and then a brief S1 nuclease digestion as described [50]. After purification, the cDNA was processed through the Ovation Ultralow DR Multiplex Systems 1–8 and 9–16 (NuGen) for end repair, barcoding and final library production. Barcoded libraries were pooled, loaded on flowcells and sequenced with Illumina HiSeq 2500 in rapid run mode to generate 50 nt single end reads. To enhance the effectiveness of cluster identification algorithm, the samples were loaded at 65% of optimal loading concentration, along with PhiX Control library (Illumina) – adapter-ligated library obtained from randomly sheared PhiX DNA – added at 10% of loading concentration to increase read sequence complexity. After an initial analysis for differential gene expression, all libraries except the five BPA treatment libraries were submitted for additional sequencing (initial comparison of sequencing data for BPA and Vehicle libraries returned no significantly differentially expressed genes). The total numbers of PF (passed-filter) reads ranged from 27.7M to 68.0M for the re-sequenced libraries and 11.9 to 18.3 for BPA libraries (Table S1). Fraction of Q30 bases ranged from 90.5% to 97.7% and average Q from 36.9 to 38.4. Sequence data will be available in Gene Expression Omnibus (Gene Expression Omnibus accession number GSE86939) and at our Primate Embryo Gene Expression Resource (www.preger.org).

RNAseq data analysis and Ingenuity Pathway Analysis

To eliminate effects of contamination from mouse embryo fibroblast feeders, reads were aligned using TopHat2 [51] to both rhesus monkey genome (MacaM v7, [52]) and mouse genome GRCm38. Reads for which the alignment score to mouse genome was higher than the alignment score to rhesus monkey genome were removed. The fraction of such reads was <20% for all libraries except one DEHP treatment library (61%), which was removed from further analysis. Reads aligned to ribosomal RNA (rRNA) or rRNA-like genes were removed. A total of 7.1M to 17.5M reads were successfully aligned to unique non-rRNA gene transcript sequences (2.5M to 4.8M for BPA treatment libraries). Cuffdiff [53] was used for quantification and differential expression analyses between the group of vehicle libraries and five groups of chemical treatment libraries; genes with q-value (false discovery rate) below 0.05 were considered differentially expressed (DE) genes (DEGs).

The results of Cuffdiff analyses were uploaded into QIAGEN Ingenuity Pathway Analysis® (IPA) and subjected to Core Analysis focusing on DE genes (q 0.05), in particular Canonical Pathway (CP) analysis, Disease and Functions (DF) analysis, Upstream Regulator (UR) analysis, and Network analysis. IPA calculates overlap p-values for each CP, taking into account the number of DE genes and the number of all molecules in knowledge database that are implicated in that pathway, as well as the number of DE genes and the

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number of molecules in knowledge database. Similarly, p-values are calculated for DFs based on the number of DE genes implicated in increase or decrease of disease or function, as well as for URs based on the number of DE genes regulated by an UR. In addition to overlap p-values, z-scores are calculated for CPs, DFs, and URs. Z-score is based on how many DE genes' direction of change (upregulation or downregulation) is consistent with activation ($z>0$) or inhibition ($z<0$) of CPs and URs, and with increase ($z>0$) or decrease ($z<0$) of DFs. Since $P(|z|>1.96)\sim 0.05$ for normal $N(0,1)$ distribution, we consider CPs, URs and DFs with $z>1.96$ to be significantly activated or increased, and those with $z<-1.96$ to be significantly inhibited or decreased. In addition to these analyses, IPA uses a greedy algorithm to construct networks that incorporate DE genes with some additional genes (or other molecules) where needed, in an attempt to reproduce possible mechanistic networks.

Results

The goal of this study was to assess the impact of long-term exposure to comparatively low levels of five EDCs on nonhuman primate embryonic cells in order to better understand how constant low level exposures affect developing embryos. To do this, rhesus monkey ORMES-6 ESCs were treated for four weeks and then RNA processed for analysis by RNAseq. This treatment period was selected to encompass three passages during treatment, in order to allow time for any DNA replication-dependent epigenetic changes. A long-term exposure of proliferating embryonic stem cells to comparatively low doses of EDCs should reveal potential effects of low-level, constant, environmentally relevant maternal exposure on cells that drive embryogenesis, without the complications of acute toxicity, maternal organ damage, or indirect maternal endocrinological effects that accompany single acute treatments with higher concentrations. A single concentration was selected for each compound due to assay and culture costs. No changes were noted in growth rate or morphology characteristics of ESC colonies during treatment. Hence, the goal of subsequent analysis was to determine effects of low-level chronic exposure on stem cell gene expression profile, and whether those changes include genes that affect stem cell function.

The number of genes with significantly (p 0.05) affected expression was modest, ranging from zero (BPA) to 119 (TBT) (Fig. 1). Between two and four times as many genes displayed decreased transcript abundance as those showing increased transcript abundance. For BPA, five genes were affected at the confidence level of $p < 0.08$. Thus, although BPA interacts with nuclear receptors, is an estrogen mimic and anti-androgen, and inhibits PPARγ, thyroid, and glucocorticoid signaling, among other effects [54], there was little effect of treatment detected here, and BPA was not studied further. The maximum fold-change values by treatment ranged from 2.66 (ATR) to 8.50 (DEHP) (Supplemental Tables S2 – S5).

Atrazine

ATR acts as a G-protein coupled estrogen receptor agonist, induces oxidative stress, and disturbs calcium homeostasis [55, 56]. ATR treatment of ORMES-6 ESCs increased the expression of 17 genes (Table S2); of these 13 were elevated by at least 1.5-fold, and five by at least 2-fold. ATR decreased the expression of 38 genes (Table S2); of these 19 were

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reduced by at least 1.5-fold, five by 2-fold, and 1 by 4-fold. The most highly up-regulated mRNAs included *FOS* (2.66-fold), *GSTK1* (2.43-fold), *FAIM* (2.29-fold), *FOSB* (2.28-fold), *GOS3* (2.03-fold) and *JUNB* (1.95-fold). Early growth response genes *EGR1* and *EGR3* were also affected. Most highly down-regulated mRNAs included *CCDC184* (4.94-fold), *PTX3* (3.7 fold), and *CLDN14* (2.86-fold).

The IPA analysis for effects of ATR treatment yielded 111 significantly affected (p 0.05) biological functions/diseases with four or more affected genes and 20 with ten or more affected genes (Table S6). The analysis returned significant z-scores (|z|>1.96, equivalent to activation p .05) for eight biological functions (Table S6) (Table 1). Functions showing activation (z>1.96) included proliferation and growth-related categories (e.g., colony formation, proliferation of connective tissues cells, colony formation of tumor cells, and fibroblast proliferation), and other functions including development of body trunk, cellular homeostasis, and Ca^{2+} flux. Cell growth and proliferation was also the top-rated affected network appearing in the IPA Network analysis (Table S7) and the UR analysis returned significant activation z-scores for *EGF*, *HMGA1*, *PDGF* and *VEGFA*, and *FOXO3* signaling (Table S8)(Table 2). Significant positive z-scores were also obtained for several cytokines. UR analysis also indicated increased activity for cellular responses to cycloheximide, indicating a possible effect on protein synthesis. The biological functions of tumor cell adhesion, cell movement and necrosis also displayed high activation z-scores, though these fell just below the significance threshold (1.96), indicating possible activation. These processes were repeated amongst similar function categories with lesser activation z-scores. One biological function (secretion) displayed a significant negative z-score, and three other transport-related biological functions yielded strong negative effects falling just below the level of significance (monosaccharide uptake, molecular transport). Apoptosis and glycolysis were among other function categories appearing repeatedly with lesser magnitude negative z-scores. The activation of *FOXO3* may contribute to this response. Significantly affected canonical pathways (CPs) with the largest numbers of affected genes included *AMPK* signaling and *NRF2* mediated oxidative stress response (four affected genes each), and GNRH signaling (three affected genes) (Table S9). Two additional stress-related CPs, *HIF1A* signaling and UVA-induced *MAPK* signaling, were affected. For comparison, past studies reported that ATR at 60 nM inhibits mouse T-cell proliferation [57], whereas 30 μM ATM does not inhibit proliferation but induces CYP19 activity in human placental cells [43].

DEHP

DEHP can act through multiple mechanisms, with effects as an anti-androgen and inhibitor of steroidogenesis, and may increase expression of DNA methyltransferase genes leading to DNA hypermethylation [58, 59]. DEHP treatment increased the expression of 12 genes (9 1.5-fold, 5 2-fold, and 2 4-fold) and decreased the expression of 48 genes (15 1.5-fold, 4 2-fold and 1 4-fold) (Table S3). The most highly upregulated genes were *RL4D* (8.5-fold), *FAIM* (4.61-fold), *GSTK1* (3.59-fold), and *PARP12* (2.42-fold). The most strongly downregulated genes were *MKRN3* (6.03-fold), *CDC184* (3.26-fold), *SPP1* (2.22-fold), and *ADM* (2.01-fold).

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IPA analysis of biological functions/diseases significantly ($p < 0.05$) affected by DEHP yielded 172 with four or more affected genes, and 33 with 10 or more affected genes (Table S10). Affected functions/diseases included just two with significant positive z-scores indicating increased activity (lung damage and tumor size) (Table 1), with additional large activation z-scores for bone size, fibrosarcoma cell death and metastasis (Table S10). No significant negative z-scores indicating inhibition were obtained, but functions/diseases with large negative z-scores below the significance threshold included tumor growth, cell spreading, cell migration and carbohydrate metabolism. Cell movement was a component of the two top-rated affected networks (Table S7). These potential negative effects on growth, spreading and migration are somewhat opposite the effects returned for ATR. CP analysis revealed a pathway related to ESC pluripotency that includes two genes downregulated by DEHP treatment (Table S11). UR analysis revealed significant inhibition of stress response mediators (*NUPR1*, *HIF1A*, *CAT*, *RLEA*, *AKT*, *p38*/*MAPK*, *MYC*, *TP53*) and infection/inflammatory response (*IFNG*, *IL5*, *IL1B*, *IFN*) and inhibition of response to chemical stress-inducing agents (e.g., peroxide) (Table S12)(Table 2). A significant activation z-score was obtained for the anti-inflammatory mediator *KLF2*.

PFOA

PFOA is a PPARα activator, and modifies genes that function in steroidogenesis, leading to diverse endocrine effects [60, 61], negatively affects thyroid function, and leads to an oxidative stress response and inflammatory response [62]. PFOA treatment increased the expression of 12 genes (11 1.5-fold and 6 2-fold) and decreased the expression of 43 genes (18 1.5-fold, 5 2-fold, and 1 4-fold) (Table S4). The most highly upregulated genes included *GSTK1* (2.87-fold), *FOS* (2.62-fold), *FAIM* (2.6-fold), *PARP12* (2.05-fold), *FOSB* (2.03-fold), and *TRIB3* (2.02-fold). The most strongly downregulated genes were *CDC184* (5.42-fold), *NRN1* (3.15-fold), *PTX3* (2.63-fold), *MKRN3* (2.59-fold), *CLDN14* (2.45-fold).

IPA analysis of biological functions/diseases significantly (p 0.05) affected by PFOA treatment yielded 140 with four or more affected genes, and 30 with ten or more affected genes (Table S13). The affected functions/diseases included four with significant z-scores indicating increased activity (cellular infiltration, injury, necrosis and hypertrophy), with additional high positive z-scores for categories related to cell death and cell movement (Table 1). One function (cell viability) had a high negative z-score value near the threshold of significance and indicating likely inhibition, consistent with the activation of cell death categories. Additional functions with z-scores indicating possible inhibition included cell protrusion, organization of cytoplasm, protein metabolism and synthesis, growth, molecular transport, cell proliferation (largest number of affected genes), and microtubule dynamics. The most significantly affected CP (5 affected members) was *NRF2*-mediated oxidative stress response (Table S14). Additional indications of changes in genes related to stress response were seen in the UR analysis (Table S15)(Table 2). Highly negative z-scores indicative of inhibition were reported for several key stress response mediators including *HIF1A*, *AKT*, *NUPR1*, *MYC*, *ERBB4*, *NOTCH1*, *ARNT*, and *SREBF1*. Additional high inhibition z-scores falling below the significance threshold were obtained for *FOS* (itself upregulated), *SMARCA4*, and *EPAS1* and for several inflammatory mediators (*IL10*,

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NEDD9, *IL5*). A significant positive z-score was also obtained for the anti-inflammatory mediator *KLF2*.

A significant positive z-score was obtained for resveratrol, also consistent with activation of oxidative stress response pathway. Positive z-scores for cycloheximide (protein synthesis inhibitor) and the RAS inhibitor Salirasib indicate potential activation of the ER stress response pathway (RAS inhibits ER stress response; [63]), again indicative of a possible inhibitory effect on protein synthesis.

TBT

TBT is an RxR/PPARγ agonist, mitochondrial ATP synthase inhibitor, a possible obesogen, and may cause DNA hypomethylation at some genes [6, 64]. TBT treatment yielded the largest number of affected genes, increasing the expression of 36 mRNAs (28 1.5-fold, 8 2-fold and 1 4-fold) and decreasing expression of 83 mRNAs (52 1.5-fold, 17 2-fold, 3 4-fold) (Table S5). The most highly upregulated mRNAs included *HOX2* (4.98-fold), *ABCA1* (3.78-fold), *CER1* (2.61-fold), *FOS* (2.44-fold), *FOSB* (2.43-fold), *SLC25A20* (2.17-fold), *LEFTY1* (2.03-fold) and *RNF219* (2.0-fold). The most highly downregulated mRNAs included *CDC184* (8.34-fold), *CLDN14* (4.84-fold), *PTX3* (4.53-fold), *RIMKLA* (3.51-fold), and *NRN1* (3.36-fold).

IPA analysis yielded 161 biological functions/diseases with significant (p 0.05) effects containing four or more affected genes (Table S16), and 68 with 10 or more affected genes. Five functions/disease were assigned significant high positive z-scores indicating increased activity: three related to organismal development (including hematopoiesis and lymphopoesis) and two related to DNA and protein binding (Table 1). Networks (Table S7) reiterated this effect on embryonic development of heart, nervous system, and hematological system. Additional biological functions with high positive z-scores included locomotion, differentiation, and transcription. Neoplasia and cancer comprised the top three functions/disease with significant or nearly significant negative z-scores indicating inhibition, followed by cancer, steroid secretion and cell survival. CP analysis revealed effects related to *TNFR*, *BMP* and *TGFB* signaling as well as effects on ES cell transcriptional regulation (Table S17).

UR analysis of TBT effects predicted significantly increased activity for signaling via *IL10*, *ESR2*, *EPO*, growth hormone, *CREBBP*, and *CSF3*, and inhibition of signaling via *HIF1A*, *STAT4*, *CD38*, *NUPR1*, *AKT*, *BMP4*, *EPAS1*, *NOTCH1*, and possibly *NEDD9* (Table 2, Table S18). A number of chemical mediators also emerged from the analysis with significant predicted increases in associated cellular activities, including Salirasib, cycloheximide, lithium, and trichostatin A.

One other notable effect of TBT was to increase expression of genes associated with controlling ESC renewal and germ layer and axis formation (LEFTY1, LEFTY2) [65, 66]. DUSP2, another regulator of cellular potency [67], was also elevated. Also elevated were BMP4, which induces primitive endoderm in monkey ESCs [68], and the hypoblast marker GATA4. Additionally, NANOG emerged as an upstream regulator of six affected genes.

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Pluripotency also appeared among affected functions for TBT (Table S16) and canonical pathways for DEHP and TBT (Tables S11, S17).

Overlap in effects

There was considerable overlap in differentially expressed gene (DEG) lists between EDCs (Tables 3 and 4). For each gene that was significantly affected by multiple toxicants, those effects had the same directionality, i.e. no gene was found to have expression increased by one toxicant and decreased by another toxicant. Affected genes showing increased expression in treated cells across three treatments included *FAIM*, *GSTK1, EGR1, FOS,* and *FOSB.* Additional genes showing increased expression shared across two treatments included *ZGRF1, TRIB3, EGR3, EPB4.1L4A, PARP12, RPUSD3,* and *ZNF280D*. Eight genes showed decreased expression across all four treatments (*ANKRD1, CCDC184, EGLN1, HK2, KDM3A, RIMKLA, SLFN5, STC1*). Between 8 and 23 genes were affected in common among different combinations of three treatments. All five genes marginally affected ($p < 0.08$) by BPA (*FOS, FOSB, EGR1, HIST1H4C,* and *HIST1HA2C*) were affected in at least one of the other treatments and all but *HIST1HA2C* were affected in two or more of the other treatments.

Substantial overlap was also seen between treatments examining results of the IPA CP analysis. To identify the most highly shared IPA CPs, we summed the $-\log_{10}(p)$ values for the four treatments and ranked the pathways accordingly (Table 5). Top rated CPs included seven affected across three treatments and 22 affected across two treatments. Notable CPs included several stress response pathways, involving, for example, *NRF2*, *AMPK*, *TNRF1*, *TNRF2*, and *HIF1A* mediators (affected by two treatments), and UVA response (affected by three treatments).

We then examined overlap among affected biological functions/diseases. The largest numbers of shared effects were seen between ATR and PFOA, and between PFOA and TBT (Table S19 and S20). One IPA function (development of body trunk) was significantly increased ($z>1.96$) for two treatments (ATR and TBT). Other IPA functions displayed trends toward shared increases or decreases across two or three treatments (e.g., $|z|\ \ 1.5$). Sorting the results by the absolute value of the sum of z-scores yielded highest positive total z-scores (n = number of treatments with $|z|\ \ 1.5$) for transcription (n=3), and tumor cell death (n=2), and strongest negative z-score sums for tumor growth (n=3), molecular secretion (n=3), molecular transport (n=2), tumor cell proliferation (n=2), and tumor cell viability (n=2) (Table S20).

UR analysis also revealed extensive overlap between treatments (Table S21) with greatest number of shared regulators between PFOA and TBT comparing any two treatments. Three regulators (*HIF1A, NUPR1* and *AKT*) displayed significant ($|z|>1.96$, $p < 0.05$) inhibition for DEHP, PFOA, and TBT treatments and a strong negative effect for ATR (Table S22, S23). *EPAS1* was inhibited in two treatments (ATR, TBT). Ranking effects according to the absolute value of the sum of z-scores (Table S23) yielded strong activation results (positive sum of z-scores) for chemical mediators (four treatments--cycloheximide, Salirasib, fulvestrant, and Pkg) and signaling ligands (two treatments--*PDGF*, *EPO*, *KLF2*). Strongest inhibition results (negative sum of z-scores) were obtained for stress response regulators:

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four treatments (*HIF1A*, *NUPR1*, *AKT*), three treatments (*CD38*), and two treatments (EPAS1, NOTCH1). Additional strong effects (sum z-scores) for two or more treatments but falling below the significance threshold were seen for NEDD9, IL5, MYC, 25-hydroxycholesterol, VEGFA, RELA, PDGF, RELA, and EPO (Table S23). There were a few instances of strong opposite effects amongst treatments; one example was *CREB1* with a strong positive activation z-score for ATR but weak negative z-score for DEHP.

Examination of the sum of -log10(p) values (Table S23) confirmed shared significant effects for upstream regulators HIF1A, NUPR1, AKT, EPAS1, NEDD9, EPO, VEGFA, IL5, Pkg, and the chemical regulators Salirisab, cycloheximide, lipopolysaccharide. Additional biological regulators emerged high in that ranking as well, such as MARCH2, ARNT, ADRA1, TNF, PDGFBB, TGFB1, IFNG, NRG1, ELK4, COMMD1, and TP53. Pathways regulated by FOSB and ADM displayed more intermediate p-value sums. Additional strong shared effects were seen for developmental regulators NANOG, GATA4, and BMP4 and weaker shared effects for SOX2, SOX17, POU5F1/OCT4.

Discussion

This is the first analysis of long-term EDC effects in a nonhuman primate embryonic stem cell model. This study reveals four striking observations. Firstly, the number of affected genes is small, ranging as high as just 119. Secondly, while there are some genes affected by just one of the EDCs tested, there is considerable overlap in affected genes among the five EDCs. Thirdly, IPA analysis indicated that long-term treatment with four of the EDCs negatively affects indicators of cell survival, as well as repressing pathways that lie downstream of several stress response mediators. And fourthly, TBT in particular, but also ATR and PFOA treatments modulated genes related to maintaining stem cell potency and cell lineage formation. Canonical pathway analysis for DEHP also indicated possible effects on pluripotency. Thus, all four of these EDCs may alter the balance between proliferation and differentiation, and predisposition for differentiation along certain embryonic lineages.

The small number of genes affected by five different EDCs applied over a four-week period is striking. The modest effect of these EDCs may be a reflection of maintaining the ESCs in a non-differentiated state; further differentiation may be required to develop the endocrine systems that these chemicals disrupt [33, 69, 70]. In studies involving induction of differentiation events from embryos or stem cells, prolonged treatment of up to two weeks in vitro revealed similar small numbers of genes induced or repressed by EDCs [71]. The numbers of affected genes can be much higher for specialized somatic cell lines [72]. In studies of ES or pluripotent cell exposures to higher EDC doses for short periods of 72 h or less, larger numbers of affected genes are seen for some EDCs [73–75], but small numbers for others [75]. The small number of affected genes for all five EDCs seen here may indicate an adaptive response of rhesus monkey ESCs occurring with chronic exposure over a protracted period of four weeks. The ability of ESCs to adapt in this manner, and manifest a limited range of genes with altered expression profiles indicates that ontogenetic effects of chronic exposure to environmental EDCs may be more selective than previously suspected. Consequently, extensive short-term responses to acute exposures affecting thousands of genes may obscure the more selective cellular and developmental consequences of low-level

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chronic exposures. Focusing attention on the small subsets of genes affected by long-term exposure provides a path to a more specific understanding of the developmental consequences and mechanisms of actions of EDCs present chronically in the environment, relevant to the effects of prevailing environmental exposures, as compared to acute short-term higher concentrations.

The genes affected by chronic exposure to a single compound tested here may provide particular targets of interest for understanding the specific effects of individual compounds (or related families of compounds) on developing embryos. Other studies using mouse ESCs, with or without induction of differentiation to various lineages, but with elevated exposure concentrations for shorter time periods, have indicated gene sets that appear to be characteristic of individual chemicals or chemical families [74], particularly with respect to interference with in vitro differentiation [76–78]. Additionally, analyses of affected biological functions and pathways, as done here, have been suggested to be key in developing biological signatures for exposures to individual chemicals or chemical families. The most highly affected genes observed here do not overlap with published gene lists that emerged as potential biomarkers using mouse ESCs treated with EDCs [74]. Only three genes affected by TBT (*KDM4B, CER1, CYP26A1*) were reported as affected in mouse ESCs by multiple other EDCs [73, 77]. The difference between the studies employing mouse versus nonhuman primate ESCs in terms of numbers of affected genes could reflect differences in chemical used, duration of treatment (e.g., 10 days), and use of a cell differentiation protocol, concentration (e.g., ID50 1.35 mM for monobutyl phthalate giving 50% inhibition of differentiation, [79]), and gene expression assay and data analysis methods, or could reflect a more fundamental species difference as previously suggested [21]. Further studies would be needed to address these possibilities.

The genes identified here as being affected by two or more separate treatments could reflect common cellular responses to harmful chemicals, or they could represent genes for which embryonic chromatin states are especially susceptible to disruption by a range of environmental factors. A better understanding of the epigenetic regulation of these genes, how their epigenetic states change during normal development, and how these epigenetic states may change in response to stress could provide a new basis for assessing whether certain developmental stages or target embryonic organs are at enhanced risk from exposure to a range of environmental agents, and may provide valuable new targets for biomonitoring.

While there was considerable overlap, there were still differences in effects of the EDCs. These differences may be related to differences in the EDCs' mechanism of action, or differences in prevalence of downstream mediators of their actions. Understanding how stem cells may modulate downstream pathways responsive to different chemical families will be valuable for understanding effects of chronic low-level exposure.

A deeper understanding of chronic exposure effects and of windows of sensitivity for different developing organs may emerge from identifying the developmental events and stages that may be most highly impacted by the biological functions and processes identified here. The subtle effects seen here on genes related to cellular proliferation and viability, and body trunk development provide potential new connections for assessing potential effects of

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chronic embryonic and fetal exposure on growth, contribution to congenital malformations, and subtle effects on neurological, immunological, and other functions. The UR analysis revealed potential compensation as part of cellular adaptation to the treatments, which could impact cell proliferation and other functions. For example, the *FOS* mRNA was upregulated in three of the treatments, but a negative activation z-score was seen for *FOS* downstream targets for ATR (−1.39) and PFOA (−1.96), indicating inhibition of *FOS*-regulated downstream functions, and no activation z-score was seen for other treatments.

Two of the EDCs (ATR and PFOA) were previously associated with an activation of stress response. The suppression of stress response pathways here with long term exposure may represent an adaptation of embryonic cells enabling their survival, and may be a general response to a variety of EDCs. The long-term inhibition of the stress response pathways could pose a special risk for developing systems, with far-reaching consequences. Such inhibition could increase the potential for developmental failure following imposition of any additional stressor. Additionally, early embryonic responses to stress are increasingly appreciated for their potential connections to diseases later in life. A variety of stressors (osmotic, nutrient, temperature, hypoxia, hyperoxia, hyperglycemia) lead to long-term effects on gene expression programming in the early embryo, with subsequent effects on progeny phenotype, and even transgenerational effects in subsequent generations [80–84]. An essential benefit of cellular stress responses is to minimize cellular damage to allow cells to recover, and protects them from undergoing necrosis or apoptosis. But even in surviving cells, sub-lethal cytological or genetic damage may occur, leading to changes in mitochondrial characteristics, cellular metabolism, DNA damage, and incorrect epigenetic programming. Suppression of stress response pathways could shift the balance to greater cell death. Indeed, we observed activation of cell death and apoptosis pathways, and reduced cell viability pathways across treatments. Increased stem cell death could reduce the numbers of stem cells available for organogenesis and tissue homeostasis in the embryo, affecting later health. Additionally, inhibition of stress response could lead to a greater degree of sub-lethal cellular damage, leading to propagation in the embryo of cells harboring damaged mitochondria or incorrect epigenetic programming.

Overall, the data presented here illustrate the need for further studies of EDC effects on development using nonhuman primate models. The dynamic pharmacokinetics of EDC metabolism during primate pregnancy [85], effects of EDCs on the uterus [86] and fetal lung [44], and the accessibility for study of nonhuman primate oocytes and embryos as a highly related animal model of human reproduction highlight the value and feasibility of using nonhuman primates to understand exposure risks to human pregnancies. Mechanistic understanding of these effects can be facilitated by determining specific effects of long-term EDC exposures on nonhuman ES and pluripotent cells, and on stem cell commitment and differentiation during successive developmental windows of susceptibility.

Although the study here does not extend to functional effects beyond gene expression changes, the data provide a foundation for further testing of effects of these low-level exposures on stem cell proliferation and developmental potential. Additionally, combinatorial treatments and low-level dose response studies would be valuable for addressing chemical mixture effects and possible additive or synergistic developmental

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effects of these chemicals in the environment. The effects reported here for cell survival, stress responsiveness, and developmental gene expression warrant further study to assess potential impact of low level chronic EDC exposure on early development in primates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATR	Atrazine
BPA	bisphenol A
CP	canonical pathway
DEG	differentially expressed gene
DEHP	di-(2-ethylhexyl) phthalate
DF	disease and function
EDC	endocrine disrupting chemical
ESC	embryonic stem cell
IPA	QIAGEN Ingenuity Pathway Analysis®
PFOA	perfluorooctanoic acid
RNAseq	RNA sequencing
TBT	tributyltin
UR	upstream regulator

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Highlights

- Effects of chronic exposure to low concentrations of 5 EDCs were examined in rhesus monkey embryonic stem cells
- There was no apparent gross change in cell morphology or growth in culture
- RNAseq revealed small numbers of affected genes
- Effects included suppression of genes related to cell survival, increased activity of cell death-related genes, suppression of downstream mediators of several stress response mediators, and modulations of pluripotency and lineage controlling genes

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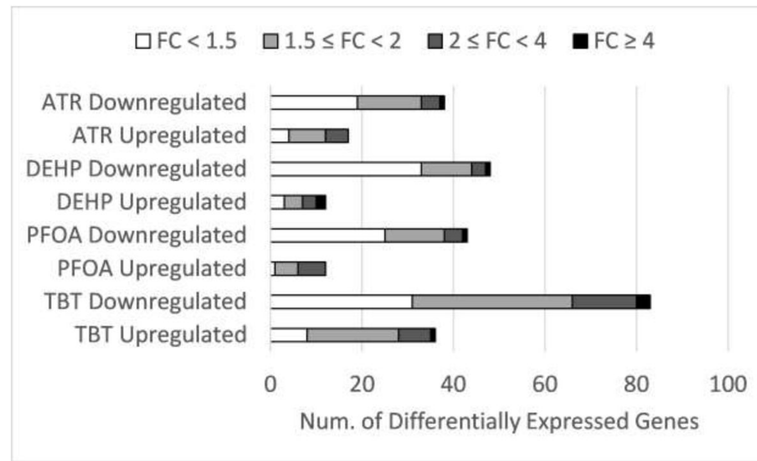

Figure 1.

Summary of differential expression in EDC treated samples. Bar segments illustrate the numbers of genes displaying fold change values in expression indicated.

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Table 1

Increased and Decreased IPA® Diseases and Functions

Toxicant	Diseases or Functions	Predicted Activation State	z-score	Num. of DEGs
ATR	colony formation of cells	Increased	2.49	7
	proliferation of connective tissue cells	Increased	2.27	6
	development of body trunk	Increased	2.20	7
	cellular homeostasis	Increased	2.13	9
	colony formation of tumor cell lines	Increased	2.13	5
	secretion of molecule	Decreased	−2.00	5
	cell proliferation of fibroblasts	Increased	2.00	4
	flux of Ca2+	Increased	1.98	4
DEHP	damage of lung	Increased	1.97	4
	size of tumor	Increased	1.96	4
PFOA	cellular infiltration by leukocytes	Increased	2.21	5
	injury of lung	Increased	1.98	4
	necrosis of liver	Increased	1.98	4
	Hypertrophy	Increased	1.98	5
TBT	development of body trunk	Increased	2.35	13
	development of hematopoietic system	Increased	2.22	7
	development of lymphatic system	Increased	2.15	8
	binding of DNA	Increased	2.10	12
	binding of protein binding site	Increased	2.08	7
	neoplasia of cells	Decreased	−2.07	16

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Table 2

Predicted Upstream Regulators

Toxicant	Upstream Regulator	z-score[1]	Num. Target DEGs
ATR	cycloheximide	2.59	8
	EGF	2.57	7
	HMGA1	2.24	5
	atorvastatin	2.24	5
	Pdgf (complex)	2.22	5
	fulvestrant	−2.21	5
	VEGFA	2.03	7
	Pkg	2.00	4
	dalfampridine	2.00	4
	FOXO3	2.00	5
	amphetamine	1.99	4
	kainic acid	1.99	4
	Pka	1.99	4
	salirasib	1.98	4
	EPAS1	−1.98	6
	MAPK3	1.98	4
	CREB1	1.98	7
	CD38	−1.98	4
	EPO	1.97	5
	CSF3	1.97	4
	LIF	1.97	4
	Akt	−1.96	4
DEHP	AGT	−2.87	10
	lipopolysaccharide	−2.73	13
	NUPR1	−2.63	7
	HIF1A	−2.53	13
	IFNG	−2.48	15
	IL5	−2.45	6
	E. coli B5 lipopolysaccharide	−2.43	6
	RELA	−2.43	6
	SREBF1	−2.37	7
	progesterone	−2.27	8
	fluticasone	−2.24	5
	fulvestrant	−2.21	5
	LEP	−2.21	6
	hydrogen peroxide	−2.21	6

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Toxicant	Upstream Regulator	z-score[1]	Num. Target DEGs
	CTNNB1	−2.19	5
	Mek	−2.19	5
	SB203580	2.19	5
	CAT	2.18	5
	Ins1	−2.18	5
	N-acetyl-L-cysteine	2.18	5
	PDGF BB	−2.15	8
	deferoxamine	−2.05	10
	TP53	−2.05	17
	IL1B	−2.04	9
	LY294002	2.01	11
	Akt	−2.00	8
	Ifn	−2.00	5
	trovafloxacin	−2.00	4
	KLF2	2.00	4
	mifepristone	1.99	5
	FSH	1.98	6
	raloxifene	−1.98	4
	TGFBR2	−1.97	4
	P38 MAPK	−1.97	5
	LDL	−1.97	4
	estrogen	−1.96	6
	paclitaxel	−1.96	6
PFOA	HIF1A	−3.19	15
	salirasib	2.81	8
	Akt	−2.59	7
	NUPR1	−2.50	13
	CD38	−2.43	6
	MYC	−2.35	9
	fulvestrant	−2.22	5
	cobalt chloride	−2.20	5
	cycloheximide	2.00	9
	Pkg	2.00	4
	ERBB4	−2.00	4
	resveratrol	2.00	5
	KLF2	2.00	4
	metribolone	−1.98	5
	NOTCH1	−1.98	4
	ARNT	−1.97	7

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Toxicant	Upstream Regulator	z-score[1]	Num. Target DEGs
	SREBF1	−1.96	6
TBT	IL10RA	2.65	7
	salirasib	2.63	7
	HIF1A	−2.62	21
	CD38	−2.62	7
	cycloheximide	2.39	7
	cobalt chloride	−2.22	5
	ESR2	2.22	9
	STAT4	−2.21	5
	NUPR1	−2.20	13
	lithium chloride	2.20	6
	trichostatin A	2.20	7
	EPO	2.19	6
	Akt	−2.19	5
	Growth hormone	2.18	5
	BMP4	−2.12	6
	EPAS1	−2.01	13
	ACVR1C	2.00	4
	Pkg	2.00	4
	COMMD1	2.00	4
	F3	2.00	5
	SB-431542	−1.99	5
	di(2-ethylhexyl) phthalate	1.98	4
	NOTCH1	−1.98	5
	CREBBP	1.98	5
	CSF3	1.97	4

[1]Upstream regulators with z-score > 1.96 are predicted to be activated, upstream regulators with z-score < −1.96 are predicted to be inhibited.

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Table 3

Differentially expressed genes affected by two or more toxicant treatments

Toxicant treatments				Common upregulated genes	Num. of genes[1]
ATR	DEHP	PFOA		FAIM, GSTK1	2 = 16.7% of 12
ATR		PFOA	TBT	EGR1, FOS, FOSB	3 = 25.0% of 12
ATR	DEHP			FAIM, GSTK1, ZGRF1	3 = 25.0% of 12
ATR		PFOA		EGR1, FAIM, FOS, FOSB, GSTK1, TRIB3	6 = 50.0% of 12
ATR			TBT	EGR1, EGR3, EPB41L4A, FOS, FOSB	5 = 29.4% of 17
	DEHP	PFOA		FAIM, GSTK1, PARP12, RPUSD3, ZNF280D	5 = 41.7% of 12
		PFOA	TBT	EGR1, FOS, FOSB	3 = 25.0% of 12
Toxicant treatments				**Common downregulated genes**	**Num. of genes[1]**
ATR	DEHP	PFOA	TBT	ANKRD1, CCDC184, EGLN1, HK2, KDM3A, RIMKLA, SLFN5, STC1	8 = 21.1% of 38
ATR	DEHP	PFOA		ANKRD1, CCDC184, EGLN1, HK2, KDM3A, RIMKLA, SLFN5, STC1	8 = 21.1% of 38
ATR	DEHP		TBT	ANKRD1, CCDC184, EGLN1, HIST1H4C, HK2, KDM3A, RIMKLA, SLFN5, STC1	9 = 23.7% of 38
ATR		PFOA	TBT	AK4, ANKRD1, CCDC184, CLDN14, EGLN1, FAM162A, HELZ, HERC3, HK2, KDM3A, NRN1, PFKFB4, PTX3, RAB2B, RIMKLA, RIOK3, SAP30, SLC2A1, SLFN5, STC1, TNIP1, UPRT, ZNF395	23 = 60.5% of 38
	DEHP	PFOA	TBT	ANKRD1, BHLHE40, BHLHE41, CCDC184, EGLN1, HK2, HMGCS1, KDM3A, PTPRB, RIMKLA, SLFN5, STC1	12 = 27.9% of 43
ATR	DEHP			ANKRD1, CAV1, CCDC184, EGLN1, GTF3C6, HIST1H2AC, HIST1H4C, HK2, KDM3A, RIMKLA, SLFN5, STC1	12 = 31.6% of 38
ATR		PFOA		ACTA1, AK4, ANKRD1, CCDC184, CLDN14, EGLN1, FAM162A, HELZ, HERC3, HK2, KDM3A, NRN1, PFKFB4, PTX3, RAB2B, RIMKLA, RIOK3, SAP30, SLC2A1, SLFN5, STC1, TNIP1, UPRT, ZNF395	24 = 63.2% of 38
ATR			TBT	AK4, ALKBH5, ANKRD1, CCDC184, CLDN14, DDX58, EGLN1, FAM162A, HELZ, HERC3, HIST1H4C, HK2, KDM3A, NRN1, PFKFB4, PTX3, RAB2B, RIMKLA, RIOK3, SAP30, SLC2A1, SLFN5, STC1, TNIP1, UPRT, ZNF395	26 = 68.4% of 38
	DEHP	PFOA		ADM, ANKRD1, BHLHE40, BHLHE41, CCDC184, EGLN1, HK2, HMGCS1, IGFBP5, KDM3A, MKRN3, PTPRB, RIMKLA, SERPINB6, SERPINE1, SLFN5, STC1, TFRC	18 = 41.9% of 43
	DEHP		TBT	ANKRD1, BHLHE40, BHLHE41, CCDC184, EGLN1, HIST1H4C, HK2, HMGCS1, KDM3A, NAMPT, PTPRB, RIMKLA, SLFN5, STC1	14 = 29.2% of 48
		PFOA	TBT	ADAMTS20, AK4, ANKRD1, BHLHE40, BHLHE41, CCDC184, CHL1, CLDN14, EGLN1, FAM162A, HELZ, HERC3, HK2, HMGCS1, KCTD12, KDM3A, LRRC58, MAP3K1, NRN1, P4HA1, PFKFB4, PTPRB, PTX3, RAB2B, RIMKLA, RIOK3, SAP30, SLC2A1, SLFN5, STC1, TNIP1, UPRT, ZNF395	33 = 76.7% of 43

[1] Fraction (%) of common genes is relative to the size of the smallest gene set included in the intersection.

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Table 4

Fold-change for genes affected by two or more toxicant treatments

Upregulated genes	ATR FC	DEHP FC	PFOA FC	TBT FC
FAIM	2.29	4.61	2.60	
GSTK1	2.43	3.59	2.87	
EGR1	1.62		1.55	1.60
FOS	2.66		2.62	2.44
FOSB	2.28		2.03	2.43
ZGRF1	1.34	1.42		
TRIB3	1.89		2.02	
EGR3	1.81			1.85
EPB41L4A	1.61			1.59
PARP12		2.42	2.05	
RPUSD3		1.87	1.70	
ZNF280D		1.57	1.66	

Downregulated genes	ATR FC	DEHP FC	PFOA FC	TBT FC
ANKRD1	1.95	1.88	1.66	1.79
CCDC184	4.94	3.26	5.42	8.34
EGLN1	1.71	1.40	1.75	2.03
HK2	1.51	1.35	1.44	1.66
KDM3A	1.49	1.38	1.45	1.65
RIMKLA	2.32	1.44	1.99	3.51
SLFN5	1.58	1.57	1.78	1.80
STC1	1.86	1.62	1.91	2.97
HIST1H4C	1.56	1.38		1.40
AK4	1.45		1.34	1.44
CLDN14	2.86		2.45	4.84
FAM162A	1.45		1.35	1.79
HELZ	1.37		1.38	1.55
HERC3	1.54		1.44	2.23
NRN1	2.76		3.15	3.36
PFKFB4	1.58		1.52	2.56
PTX3	3.70		2.63	4.53
RAB2B	1.42		1.41	1.70
RIOK3	1.43		1.39	1.66
SAP30	1.61		1.45	1.71
SLC2A1	1.58		1.54	1.70
TNIP1	1.43		1.34	1.85

Downregulated genes	ATR FC	DEHP FC	PFOA FC	TBT FC
UPRT	1.56		1.41	1.59
ZNF395	1.44		1.40	1.61
BHLHE40		1.61	1.55	1.78
BHLHE41		1.69	1.66	2.67
HMGCS1		1.49	1.44	1.42
PTPRB		1.52	1.59	1.56
CAV1	1.45	1.42		
GTF3C6	1.33	1.35		
HIST1H2AC	1.42	1.31		
ACTA1	1.39		1.38	
ALKBH5	1.33			1.39
DDX58	1.53			1.96
ADM		2.01	1.95	
IGFBP5		1.81	1.55	
MKRN3		6.03	2.59	
SERPINB6		1.70	1.44	
SERPINE1		1.50	1.47	
TFRC		1.43	1.35	
NAMPT		1.38		1.55
ADAMTS20			1.46	2.02
CHL1			1.34	1.52
KCTD12			1.45	1.61
LRRC58			1.34	1.41
MAP3K1			1.36	1.45
P4HA1			1.32	1.46

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Table 5

IPA® Canonical Pathways significantly affected by two or more toxicant treatments

	$-\log_{10}(p)$ [1]				
Canonical Pathways	**ATR**	**DEHP**	**PFOA**	**TBT**	**SUM**
Circadian Rhythm Signaling		2.41	2.48	1.84	6.73
GNRH Signaling	2.32		2.34	1.45	6.11
Virus Entry via Endocytic Pathways	1.64	2.69	1.65		5.97
PDGF Signaling	1.75	1.70	1.77		5.22
UVA-Induced MAPK Signaling	1.64	1.59	1.66		4.89
CDK5 Signaling	1.55		1.56	1.75	4.86
HIF1α Signaling	1.53	1.47	1.54		4.54
NRF2-mediated Oxidative Stress Response	2.89		3.99		6.88
Superpathway of Cholesterol Biosynthesis		2.55	2.62		5.17
AMPK Signaling	2.90		1.96		4.86
TNFR1 Signaling			2.14	2.59	4.73
Mevalonate Pathway I		3.22	1.48		4.70
TNFR2 Signaling			2.59	1.95	4.54
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)		2.98	1.36		4.35
April Mediated Signaling			2.36	1.72	4.08
Tight Junction Signaling	2.01		2.03		4.04
B Cell Activating Factor Signaling			2.32	1.68	4.00
Renal Cell Carcinoma Signaling	1.82		1.84		3.66

Canonical Pathways	$-\log_{10}(p)$ [1]				
	ATR	DEHP	PFOA	TBT	SUM
Caveolar-mediated Endocytosis Signaling	1.82	1.76			3.58
TGF-β Signaling			1.67	1.90	3.57
CD27 Signaling in Lymphocytes			2.09	1.47	3.56
RAR Activation			1.88	1.66	3.54
EGF Signaling			2.03	1.41	3.44
NAD Biosynthesis III		1.77		1.48	3.26
Salvage Pathways of Pyrimidine Ribonucleotides	1.61		1.62		3.23
LPS/IL-1 Mediated Inhibition of RXR Function			1.71	1.45	3.16
Ketogenesis		1.55	1.59		3.14
Vitamin-C Transport	1.41		1.42		2.83
Glutathione Redox Reactions I	1.31		1.32		2.63

[1] Only values -log10(p) 1.30 (equivalent to p .05) are shown in the table.

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